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***Drosophila* Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic**

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The TGF- β (transforming growth factor- β)-related signalling proteins, including Decapentaplegic (Dpp) in *Drosophila* and bone morphogenic proteins and activin in vertebrates, affect the growth and patterning of a great variety of structures. However, the mechanisms by which these ligands regulate gene expression are not understood. Activation of complexes of type I with type II receptors results in the phosphorylation and nuclear localization of members of the SMAD protein family^{1–9}, which are thought to act as co-activators of transcription, perhaps in conjunction with

sequence-specific cofactors¹⁰. Here we show that the amino-terminal domain of the *Drosophila* Mothers against dpp protein (Mad), a mediator of Dpp signalling^{11–14}, possesses a sequence-specific DNA-binding activity that becomes apparent when carboxy-terminal residues are removed. Mad binds to and is required for the activation of an enhancer within the *vestigial* wing-patterning gene in cells across the entire developing wing blade. Mad also binds to Dpp-response elements in other genes. These results suggest that Dpp signalling regulates gene expression by activating Mad binding to target gene enhancers.

In the *Drosophila* wing imaginal disc, the anteroposterior and dorsoventral compartment boundaries are important signalling sources, and inputs from both axes are required for appendage formation¹⁵. Growth and patterning along the anteroposterior axis depends upon the sequential organizing activities of the Engrailed (En), Hedgehog (Hh) and Decapentaplegic (Dpp) proteins^{16–19}. Dpp acts as a morphogen from its source to organize wing growth and anteroposterior patterning and to activate gene expression over a long range^{19–24}. The *spalt* (*sal*)²⁵ and *optomotor-blind* (*omb*)²⁶ genes are expressed in nested patterns centred on and extending up to 20 cell-diameters away from the stripe of Dpp expression^{22,23} (Fig. 1a). The *vestigial* (*vg*) gene is even more broadly expressed and is required in all cells of the developing wing (Fig. 1a). Activation of *vg* is regulated by signals from both axes through separate *cis*-regulatory elements that control complementary patterns of gene expression²⁴. The 'boundary' enhancer is activated along the dorsoventral boundary through components of the Notch pathway, whereas the 'quadrant' enhancer is activated in the remainder of the developing wing blade (Fig. 1e) by Dpp as well as a signal from the dorsoventral boundary²⁴.

Because Mad is an intracellular signal transducer downstream of Dpp receptors^{1,13,14}, we examined the requirement of Mad activity for Vg expression in the wing imaginal disc in mitotic clones with reduced Mad function. Homozygous clones for the strong *Mad*^{1,2} allele in developing wing-blade cells had significantly reduced levels of Vg expression and showed a growth disadvantage in comparison with surrounding heterozygous or wild-type cells (twin spot) (Fig. 1b–d, arrows). In contrast, *Mad*^{1,2} clones along the dorsoventral boundary did not show any changes in Vg expression levels (Fig. 1b–d, arrowheads). The different effects of the reduction of Mad activity in dorsoventral boundary versus wing-blade clones is explained by the different regulatory elements that control the expression of the *vg* gene in these regions. Mad has no effect on the Notch-dependent dorsoventral boundary enhancer, but the quadrant enhancer required Dpp signalling²⁴ and Mad function.

To investigate whether Mad exerts a direct effect on *vg* transcription, we tested whether Mad could bind specifically to the quadrant enhancer. SMAD family members share highly conserved amino- and carboxy-terminal domains, termed MAD homology regions 1 and 2 (MH1 and MH2)², which are separated by a less conserved proline-rich linker region. We found that the Mad MH1 plus linker, expressed as a glutathione S-transferase (GST) fusion protein (designated Mad^N), bound DNA and protected a single interval within the quadrant enhancer in a DNase I footprinting experiment (Fig. 2a); GST and the GST–MAD linker plus MH2 fail to bind DNA (data not shown). The specificity of Mad binding to these protected sequences was demonstrated by a gel mobility-shift assay (Fig. 2b). A 39-base-pair double-stranded oligonucleotide of the *vg* quadrant enhancer containing the Mad-protected region, the Q⁺ probe, was bound readily by Mad^N (Fig. 2b, lane 2). A double-stranded oligonucleotide in which 12 bp of the Mad^N protected region was replaced with two *Bgl*II restriction sites, the Q^m probe, was bound with much lower affinity than the wild-type sequence (Fig. 2b, lane 7). Moreover, the Q^m mutant oligonucleotide did not compete as efficiently as the Q⁺ oligonucleotide with the ³²P-labelled Q⁺ probe (Fig. 2b, compare lanes 3 and 4 with lanes 5 and 6). These data suggest that the binding of Mad^N protein to the quadrant enhancer

is specific. Only a single predominant band shift was observed, regardless of competitor concentration (Fig. 2b, lanes 2–6). These results suggest that Mad^N binds to DNA as a single species.

To test whether the Mad^N binding site is essential for the activation of the quadrant enhancer *in vivo*, we generated transgenic fly lines carrying a *lacZ* reporter gene under the control of the quadrant enhancer in which the 12 bp of the footprinted interval were replaced with one or two 6-bp *Bgl*II restriction sites, the 12-bp substitution corresponding in sequence to the Q^m oligonucleotide that fails to bind Mad^N. Normally, the quadrant enhancer is activated in early third-instar discs as a small patch of expression

near the intersection of the anteroposterior and dorsoventral boundaries and gradually fills in the entire wing pouch by the mid–late third instar²⁴ (Fig. 2c). In contrast, expression of the *lacZ* reporter gene driven by either mutated quadrant enhancer was not detectable in most early to mid third-instar discs. When detected, expression was only observed at a very low level near the antero-posterior boundary (Fig. 2d, e). These data, together with the *in vitro* data from the Mad binding experiments on the *vg* quadrant enhancer, demonstrate that Mad directly mediates the Dpp-dependent transcription of the *vg* gene.

The identification of an essential Mad binding site in the quad-

Figure 1 Mad activity is required for Vg expression in the cells of the developing wing blade. Native Vg protein expression is shown in red. **a**, Top, triple-labelled third-instar wing disc with nested domains of Spalt (purple and light blue) and Omb (green), and yellow where coexpressed with Vg expression within the broad Vg (red) domain. Bottom, these wing patterning genes are activated over different distances from the Dpp source. **b**, Mitotic clones homozygous for the hypomorphic *Mad*¹² allele lack expression of the Myc epitope tag (green). **c**, Vg expression in the same disc as **b**. **d**, Wingless (Wg) (blue) expression merged with the Vg expression (red) shown in **b** marks the dorsoventral boundary. The level of Vg expression is reduced only in the *Mad*¹² clone in the future wing blade (arrows), but not in the clones on the dorsoventral boundary (arrowheads). **e**, Vg expression (red) in the future wing-blade cells is regulated by the *vg* quadrant enhancer (β -galactosidase expression driven by the quadrant enhancer is in light blue and purplish pink).

Figure 2 The binding of Mad^N protein to the *vg* quadrant enhancer is sequence specific and essential for activation *in vivo*. **a**, DNase I footprint of the Mad^N protein to the *vg* quadrant enhancer. An autoradiogram of a denatured polyacrylamide gel used to separate the products of DNase I digestion of Mad^N-*vg* quadrant enhancer complexes is shown (right four lanes) with the DNA sequencing products of the *vg* quadrant enhancer (left four lanes, marked with their corresponding ddNTP reaction). The schematic representations of the relative amounts of Mad^N protein used in the reaction (over the right four lanes) shows that Mad^N was increased twofold in the second lane and fourfold in the third lane; the fourth lane is the no-protein control. The DNase I-sensitive bases protected by Mad^N are marked with asterisks. The double-stranded sequence spanning the protected region is shown. **b**, Competition gel mobility-shift assay for the binding specificity of Mad^N to the *vg* quadrant enhancer DNA. The presence or absence of each component is indicated by + or -; 10x and 100x indicate the relative molar excess. Q⁺ is the wild-type double-stranded oligonucleotide containing the Mad^N footprinted interval; Q^m is the oligonucleotide in which the footprinted interval has been replaced with two *Bgl*II sites (see Methods for sequences). The Q⁺ probe was bound well by Mad^N (lane 2) but the Q^m probe was not (lane 7). The unlabelled Q^m oligonucleotide did not compete efficiently for the Q⁺-Mad^N complex formation (lanes 5 and 6), whereas the Q⁺ oligonucleotide did (lanes 3 and 4). **c**, Expression of β -galactosidase driven by the wild-type *vg* quadrant enhancer in a mid–late third-instar wing imaginal disc. **d**, **e**, Expression of β -galactosidase driven by the mutated *vg* quadrant enhancer in which the 12-bp Mad binding site has been replaced with a *Bgl*II site (**d**) and two tandem *Bgl*II sites as in Q^m (**e**). The expression level of β -gal was greatly reduced in these discs compared with the wild-type enhancer (**c**). The imaginal discs in **c–e** carry one copy of the reporter gene.

Figure 3 The N-terminal domain of Mad protein (Mad^N) binds to the *vg* quadrant enhancer. **a**, MAD fusions to the C terminus of MBP or GST for use in DNA-binding assays. Conserved MH1 and MH2 regions are shown as boxes, with the intervening linker region indicated by a line. Fusion proteins contain the following MAD amino acids: B-H (Mad), 1–455; B-Bg, 1–372; B-Pv (Mad^N), 1–241; R107C, 1–241; B-E, 1–159; B-X, 1–120; B-S, 1–48; S-Pv, 49–241; Pt-H (MAD^C), 167–455. Amino-acid residue number is indicated at the top. Relative DNA binding activity is shown on the right. Protein concentrations were estimated by display on the SDS

polyacrylamide gel in **b**. Data are from **c** and two repetitions of this experiment. Standard error is indicated within brackets. **b**, Coomassie blue-stained 10% SDS gel showing affinity-purified preparations of MBP fusion proteins used in **c**. **c**, Autoradiographic image of a gel mobility-shift assay testing the ability of MBP-Mad fusions to bind to a ³²P-end-labelled 20-bp *vg* double-stranded oligonucleotide. Lane 1, no protein control; lane 24, unfused MBP; remaining sets of lanes contain progressive 5-fold increases in the proteins labelled as in **a**.

rant enhancer allowed us to localize the Mad DNA-binding domain more precisely. In a series of fusions to the maltose-binding protein (MBP) of *Escherichia coli*, the effects of deletions from the Mad C and N termini (Fig. 3a, b) on DNA-binding activity were tested using an oligonucleotide containing the Mad site of the quadrant enhancer (Fig. 3c). The specific DNA-binding activity of full-length Mad protein (B-H, lanes 2–4) was approximately 100-fold lower than that of C-terminally truncated Mad^N (B-Pv, lanes 8–10); removal of only a portion of MH2 (B-Bg, lanes 5–7) resulted in an intermediate activity. These results show that MH2 effectively inhibits DNA binding and suggests a mechanism that might contribute to inactivation of Mad in the absence of Dpp signalling. Removal of the linker region from the C-terminal end of MH1 (B-E, lanes 11–13) caused a slight reduction in binding activity. Without MH1, MH2 plus linker region (Mad^C) failed to bind to DNA (Pt-H, lanes 23–25). Removal of the C-terminal (B-X, lanes 14–16) or N-terminal (S-PV, lanes 20–22) portion of the MH1 domain abolished DNA-binding activity. Binding was also abolished by a single amino-acid substitution in Mad (Arg107 → Cys) that is the same as that found at position 133 in the Smad2 protein in a colorectal tumour⁴ (R107C, lanes 26–28), demonstrating that this conserved residue is essential for MH1 DNA-binding activity.

We also sought to determine a consensus sequence recognized by

Mad^N. As well as the *vg* quadrant enhancer, several other Dpp-responsive enhancers have been characterized, such as the *labial* endoderm enhancer^{27,28} and the *Ultrabithorax* midgut enhancer, for which the Dpp response element has been localized to the 95-bp DI–DII interval²⁹. Gel shifts with oligonucleotides representing portions of these enhancers (Fig. 4b, lanes 1–18), and the competition of Q⁺ but not Q^m for these oligonucleotides (Fig. 4c, lanes 9–12, 15–18), show that at least one high-affinity Mad^N binding site is found in each, together with multiple low-affinity sites. Alignment of these sequences and comparison of the relative strength of Mad^N binding were used to derive a Mad^N binding-site consensus of GCCGnCGC. The two sites of highest affinity match this consensus perfectly, and three lower-affinity sites contain mismatches at one to three positions (Fig. 4d).

Our results demonstrate that the Mad MH1 region has a sequence-specific DNA-binding domain, and indicate that this DNA-binding activity is important in directing transcriptional activation in response to Dpp signalling. The C-terminal MH2 region, in addition to its previously demonstrated function as a transcriptional activation domain (in the case of Smad1)³, inhibits the DNA-binding activity of MH1 *in vitro*. It is not yet known whether the masking of DNA-binding activity (in addition to cytoplasmic sequestration by receptors⁸) is involved in the regula-

Figure 4 The *lab* endodermal and *Ubx* midgut enhancers contain Mad^N binding sites similar to those in the *vg* quadrant enhancer. **a**, DNA sequences of a portion of the *lab* endodermal enhancer and of the DI and DII regions of the *Ubx* midgut enhancer, each of which contains sequences resembling the Mad binding site of the quadrant enhancer. The regions corresponding to the probes used for the gel mobility-shift assay in **b** are marked with brackets. The asterisk indicates a base pair that was omitted in the *Ubx-A* probe. **b**, Gel mobility-shift assay identifies a high-affinity Mad^N binding site in the *lab* enhancer and one in the *Ubx* DI region. The sequences of the *lab* and *Ubx* probes are shown in **a**. *vg* is a 20-bp probe containing the Mad^N binding site; *Q+* and *Q^m* are 40-bp probes containing wild-type and mutated versions of the same site. Each set of 30-μl binding reactions contained (left to right) 0, 100 and 500 ng of fusion protein, as indicated by the minus sign and wedge. **c**, Competition assays for specificity of Mad^N binding to *vg*, *Ubx-B* and *lab-C* oligonucleotides. A10- and 100-fold excess of *Q+* or *Q^m* oligonucleotide over labelled *vg*, *Ubx-B* or *lab-C* probe was incubated in binding reactions containing 250 ng of Mad^N. In each case *Q+*, but not *Q^m*, competed efficiently for binding to Mad^N showing that each site is bound with a high degree of sequence specificity. **d**, Alignment of sequences that are bound by Mad^N in **b**. The GCCGnCGc consensus derived from the aligned sequences matches the two highest affinity sites perfectly, contains one mismatch with a moderate affinity site, and contains 2–3 mismatches with two low-affinity sites.

tion of Mad activity by Dpp *in vivo*. One further mechanistic issue concerns the establishment of nested expression domains of Dpp-regulated genes in the wing. Different thresholds for response to Dpp could result from different affinities and/or numbers of Mad binding sites in target enhancers. This possibility can be tested once the Dpp-responsive *cis*-regulatory element(s) of other wing-patterning genes is isolated and characterized.

Note added in proof: Ersh *et al.* (EMBO J. 16, 2014–2022; 1997) have recently provided evidence that Dpp induces visceral mesodermal expression of *Ubx* by direct binding of dCREB B protein to a CRE element. This CRE is adjacent to a palindromic sequence, termed FP5, which footprints with an embryonic nuclear extract. FP5 is coincident with the high-affinity Mad binding site we identified in the *Ubx* B oligo. Ersh *et al.* showed that reporter gene response to Dpp was dramatically reduced by mutation of the CRE but not by mutation of FP5. Thus Dpp-dependent activation of *Ubx* may not require DNA contact by Mad, but rather by dCREB B. These contrasting results for *vg* and *Ubx* suggest that the mechanism by which Mad mediates transcriptional regulation may vary significantly among the many targets of Dpp signalling. □

Methods

Generation of Mad clones and immunohistochemistry. Mitotic clones homozygous for the *Mad*^{1,2} allele were generated by heat-shock induction of FLP recombinase in the progeny of *w; Mad*^{1,2}, P[ry+, hs-neo, FRT] 40A/CyO males (stock provided by V. Wiersdorff, M. Mlodzik and S. Cohen) crossed to *w*¹¹¹⁸, hsFLP1; P[w+, hs-pM] 36F, P[ry+, hs-neo, FRT] 40A female flies. Clone

induction and antibody staining of imaginal discs was performed as previously described²⁴.

Production and purification of Mad fusion proteins. For fusions containing the MH1 domain (Mad and Mad^N) a *Bam*HI site was introduced by polymerase chain reaction (PCR) 15 bp upstream of the predicted initiator ATG. Fusion of this *Bam*HI site to a *Bam*HI polylinker site at the C terminus of GST or MBP resulted in insertion of the sequence Gly-Ser-Ser-Asn-Arg between GST (or MBP) and MAD. The full-length Mad fusions contained Mad sequences extending from the *Bam*HI site to a *Hind*III site located within the 3' untranslated region. Truncated fusions were constructed using the restriction sites indicated in Fig. 3a. The R107C point mutation was introduced into the Mad coding region by two-primer site-directed mutagenesis, and was confirmed by sequencing. The GST and MBP fusion proteins were purified by affinity chromatography³¹.

Gel mobility shift and DNase I footprinting assays. DNA-binding assays for Mad fusion proteins were performed in binding buffer (25 mM Tris, pH 7.5, 80 mM NaCl, 35 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol (v/v), 50 μg ml⁻¹ poly(dI.dC), 50 μg ml⁻¹ poly(dA.dT), 150 μg ml⁻¹ nonspecific competitor DNA) and separated in 5% polyacrylamide gels containing 0.5 × TBE. The top strands of the double-stranded oligonucleotides used are indicated in Fig. 4 except for the following, in which underlining shows where *Q+* and *Q^m* differ in sequence: *Q+*, TTTGTGCTTGGCTGCCGTCGCGATTTCG ACAACTTTGG; *Q^m*, TTTGTGCTTGGATCTAGATCTATTGACAACCTT GG; *Vg*, CTTGGCTGCCGTCGCGATTTC. Oligonucleotide probes corresponding to regions of the *vg* quadrant, *Ubx* midgut, and *lab* endodermal enhancer sequences were labelled with [γ-³²P]ATP (6,000 Ci mmol⁻¹, NEN) and T4 polynucleotide kinase. The probe DNAs for DNase I footprinting were

synthesized by PCR. DNase I footprinting reactions were processed as described²⁴.

Reporter gene construction. Substitution of a single *Bgl*II site for the quadrant enhancer Mad binding-site sequence GCTGCCGTCGCG was done by assembling two fragments generated by PCR with *Pfu* polymerase. Expansion of the single *Bgl*II site to two tandem sites matching the Q^m sequence was done by insertion of an oligonucleotide containing *Bbs*I internal sites that allowed for subsequent cleavage and ligation to generate the tandem *Bgl*II sites. Both sequences were confirmed by sequencing and ligated into the *Kpn*I/*Not*I sites of the Hsp *lacZ*-CaSpeR *Drosophila* transformation vector for the generation of transgenic lines²⁴.

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errata

Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration

Ricardo E. Dolmetsch, Richard S. Lewis, Christopher C. Goodnow & James I. Healy

Nature **386**, 855–858 (1997)

The graphs in Fig. 2e were accidentally swapped around during the production process. The correct versions are shown here. □

Large-scale tectonic deformation inferred from small earthquakes

Falk Amelung & Geoffrey King

Nature **386**, 702–705 (1997)

Owing to an error in the production process, a length scale was omitted from Fig. 2b and the shading in Fig. 2d did not show up. The corrected figure is shown here. □